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INTRODUCTION

The main goal of the study was to test the hypothesis that a reduction in gene expression could induce gene silencing (i.e. relatively stable loss of gene expression) in breast cells. Silencing of a variety of tumor suppressor genes plays a major role in the initiation and progression of breast cancer. Our ultimate goal is to determine if environmentally induced gene repression plays a role as a trigger for the silencing of genes. The purpose of the work described below was to confirm or refute the hypothesis. The anticipated scope of the work was to test tumor suppressor promoters that become silenced in breast cancer for repression-mediated gene silencing, but we soon realized that this scope was too ambitious for a one-year funding period. We therefore changed the scope somewhat by working with a generic mouse cell line (fibroblast-like) and creating a model system to ensure that during the funding period we could at least test the basic principle of the hypothesis. This approach was successful and we have now demonstrated: 1) that gene repression can induce gene silencing in mammalian cells and that 2) this approach can be used to induce silencing of the *BRCA1* promoter.

BODY

a. Introduction-Our original plan was to obtain gene promoters for tumor suppressor genes known to be silenced in breast cancer cells, link these promoters to the selectable *HPRT* cDNA, transfect these promoters stably into *HPRT* deficient MCF-7 breast cancer cells, use environmental agents to repress promoter function (and thereby reduce HPRT protein levels), and then determine if reduced HPRT protein levels could persist in the absence of continued promoter repression. Such a result would demonstrate that gene repression could induce gene silencing in breast cells. It quickly became apparent, however, that this agenda was too ambitious for a variety of reasons including the relatively slow growth of the MCF-7 cells and the time required to clone and test the tumor suppressor gene promoters. My fear was that we would create the reagents necessary to test the hypothesis within a year, but not have sufficient time to create conditions under which we could actually conduct the test. I therefore made the decision to modify the scope of the proposed work to allow us to test the most important part of the hypothesis, which is that gene repression can induce gene silencing and then if time permitted to demonstrate that this approach could be used to silence a tumor suppressor promoter that plays a role in breast cancer. The system that was developed and the results obtained with this system and with the *BRCA1* promoter are detailed below.

b. *Repression-mediated silencing with the tet-off system*-We used the tet-off system to create a model in which expression of a target gene could be specifically repressed; in this case by exposure to doxycycline (Dox), a tetracycline analog. The

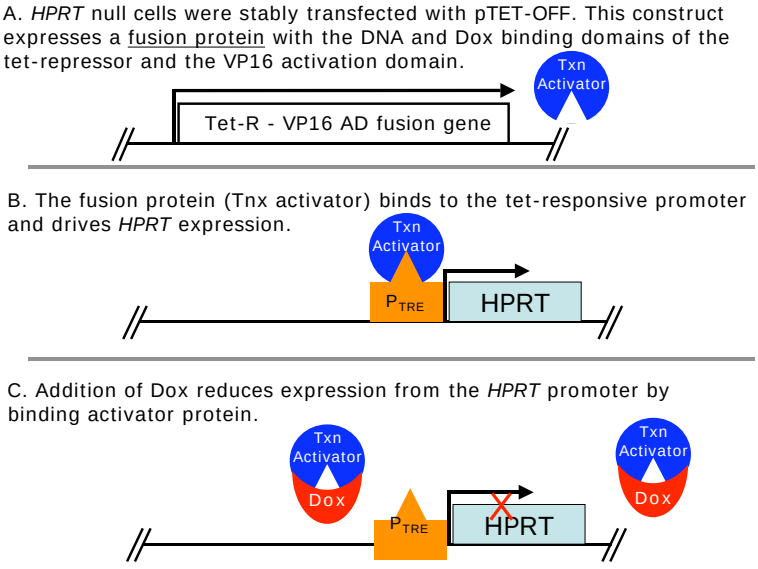


Figure 1. The tet-off system. Cells are transfected with a construct that expresses an activator protein (A) that binds to the tet response element (TRE) on a second construct that expresses HPRT cDNA (p_{tet}-HPRT) (B). Addition of Dox to the medium leads to removal of the TRE protein from the promoter, and hence a reduction in gene expression (C).

target gene in this model system remained the selectable human *HPRT* cDNA, which encodes a protein that converts hypoxanthine and guanine to IMP and GMP, respectively. *HPRT* deficient cells can be selected by adding thioguanine (TG) to the culture medium because TG kills cells that express *HPRT*, whereas *HPRT* deficient cells can grow in the presence of TG. The Dox repressible construct expressing HPRT cDNA, termed p_{tet}-HPRT, was transfected stably into a *Hprt* deficient mouse cell line termed DIF-6 that also contains the activator protein. Fig. 1 shows both constructs (1A and B) and how the system works to express *HPRT*, or to repress *HPRT* (1C) when Dox is added to the cell culture medium. Hence, Dox represses transcription of *HPRT* and removal of Dox from the medium leads to rapid restoration of *HPRT* expression.

Table 1. Induction of phenotypic gene silencing via transient repression of *HPRT* cDNA with Dox[1].

Cell Line[2]	Treatment	Silencing Freq.[3]
HPRT 1[4]	untreated	9.8×10^{-6}
HPRT 1	1 μM Dox (7 days)	9.4×10^{-3}
HPRT 3	untreated	$< 4.5 \times 10^{-6}$
HPRT 3	1 μM Dox (7 days)	2.1×10^{-4}
HPRT 4	untreated	$< 4.4 \times 10^{-6}$
HPRT 4	1 μM Dox (7 days)	1.6×10^{-4}

1 Doxycycline (Dox) represses transcription of minimal CMV promoter by removing an activating protein.
2 Each cell line represents an independent transfectant.
3 Silencing frequencies represent the fraction of thioguanine (TG) resistant clones (see text for more detail).
4 This cell line gave rise to a single spontaneous TG resistant clone.

The question we asked first was whether transient repression of *HPRT* transcription would lead to gene silencing, as predicted by the hypothesis. For these experiments, we treated three *HPRT* expressing transfectants containing the Dox repressible construct (HPRT 1, 3, and 4) for one week with 1 μM Dox. Following the one-week treatment, Dox was removed from the medium to allow *HPRT* expression to return, and then the cultures were exposed to TG. The results from a representative experiment are shown in Table 1. Although most cells died in the presence of TG after Dox was removed from the medium, which means these cells recovered *HPRT* expression, a small fraction cells became TG resistant cells due to Dox exposure at frequencies ranging from 10^{-3} to 10^{-4}. TG resistant clones were not observed in cultures that did not receive Dox treatment, with a single exception. Fig. 2 demonstrates that the frequency of TG resistant clones rose as a function of time that *HPRT* expression was repressed by Dox.

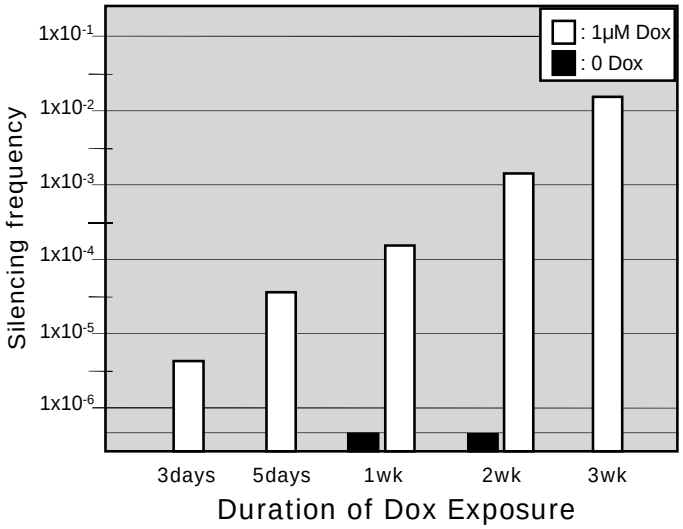


Figure 2. Silencing frequency increases as a function of Dox exposure. HPRT 3 cells (see Table 1) were exposed to Dox for the times indicated. After exposure, the cells were plated in the presence of TG to determine silencing frequencies. Cells exposed to no Dox (0 dox) for 1 and 2 weeks were also sampled, but neither plating yielded TG resistant clones.

The ability of the cells to grow into clones in the presence of TG after Dox was removed suggested that silencing occurred. An alternative explanation was loss of *HPRT* expression via mutational inactivation. The quickest way to distinguish *bona fide* mutational events from the

.

silencing process is to measure reversion frequencies, which detects cells that reacquire *HPRT* expression. Silenced alleles often revert spontaneously at high frequency, whereas mutant alleles revert at low frequency or, more often, not at all. Moreover, gene silencing is often associated with increased promoter region DNA methylation and decreased histone deactylation, which are reversible with 5-deoxyazacytine (5-aza-dC) and trichostatin A (TSA), respectively. Reversion frequencies were measure for untreated cells, cells treated with 5-aza-dC, cells treated with TSA, and cells treated with both 5-aza-dC and TSA. Fig. 3 shows that: 1) spontaneous revertants were present in the TG resistant population, 2) TSA and 5-aza-dC independently induced more revertants, and 3) these two drugs acted synergistically when combined. These results confirmed that silencing had occurred and that both promoter region DNA methylation and histone deacetylation were present at the promoter of the p_{tet}-HPRT. A bisulfite sequence analysis directly demonstrated promoter region DNA methylation in the TG resistant silenced clones, but not in parental cells even when *HPRT* was repressed in the presence of Dox (Figure 4). A Chromatin Immunoprecipitation (ChIP) analysis directly demonstrated histone lysine 9 deacetylation in the TG resistant clones, and also demonstrated reduced histone lysine 4 methylation and increased lysine 9 methylation; these alterations are associated with epigenetic silencing (Fig. 5) . Considered as a whole,

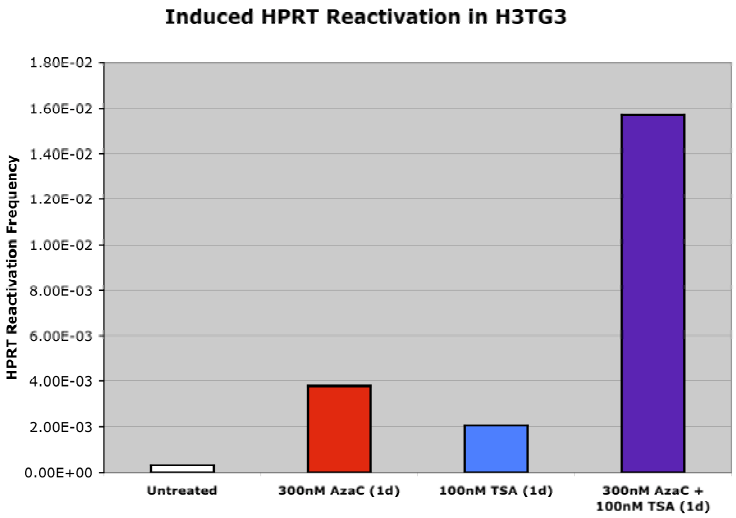


Figure 3. *HPRT* reversion frequencies for a TG resistant clone. Reversion frequencies, which detect and qunatify reactivation of silenced p_{tet}-HPRT alleles, were determined for an untreated H3TG3 clone, and after exposure to 300 nM 5-aza-dC, 100 nM TSA, and a combination of both. All treatments were for 24 hours and an additional 24 hours was allowed before selecting revertant cells.

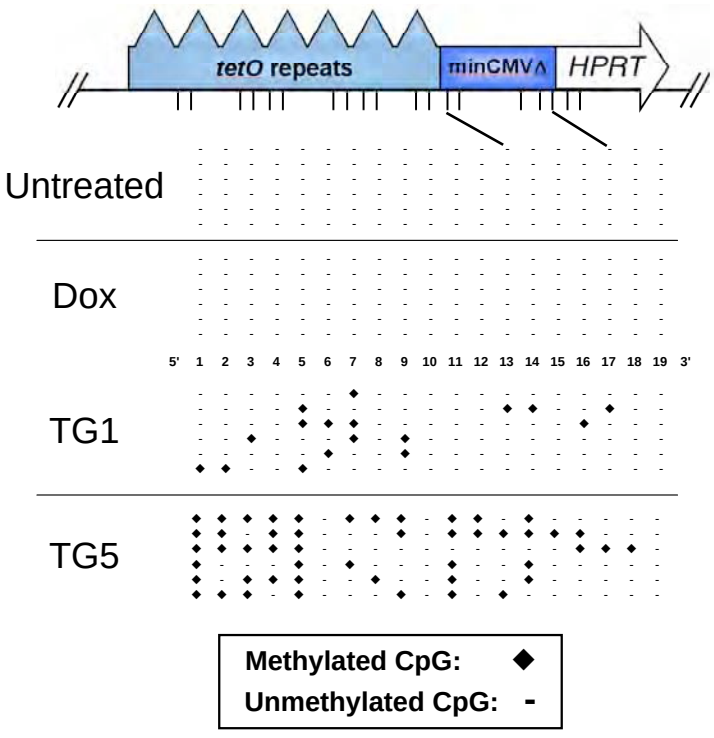
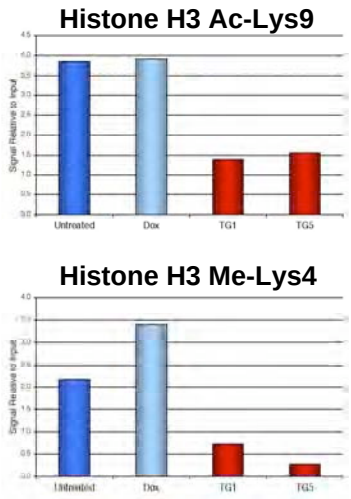


Figure 4. Promoter Region DNA Methylation Is Associated with Inactivation of *HPRT*. Bisulfite sequencing was performed for the promoter region of *p_{tet}-HPRT* in the H4-HPRT cells. Shown are results for untreated cells, cells exposed to Dox for one week, and two TG resistant clones (TG1 and TG5) isolated from Dox treated cells. The tetO repeats bind the tTA activator protein (Figure 1); minCMV represents a minimal CMV promoter. The CpG sites in CMV minimal promoter are bracketed.

these data demonstrate conclusively that a transient reduction in gene expression (brought about by Dox) induced epigenetic silencing characterized by promoter region DNA methylation and histone modification, and that silencing was distinguishable from repression.



We next asked the relative order and importance of histone acetylation and DNA methylation by repeating the experiment in which the cells were exposed to Dox for one week to induce silencing, but some dishes received 5-aza-dC or TSA for the last 24 hours of Dox exposure. Figure 6 shows that the induction of silenced clones in the presence of Dox was unaffected by 5-aza-dC, but nearly completely reversed by exposure of the cells to TSA. This result demonstrated than an early step in inducible gene silencing is histone deacetylation and that silencing does not require promoter region DNA methylation. Instead, DNA methylation is a consequence of silencing.

Figure 5. Repressive Histone Modifications Are Associated with Inactivation of *HPRT*. Chromatin immunoprecipitation (ChIP) assay was used to assess lysine 9 acetylation (Ac-Lys9), lysine 9 methylation (Me-Lys9), and lysine 4 methylation (Me-Lys4). Shown are results for untreated cells (dark blue), cells exposed to Dox for one week (light blue), and two TG resistant clones (TG1 and TG5) induced by exposure to Dox.

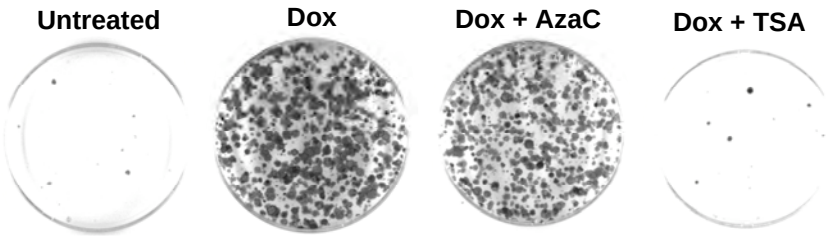


Figure 6. Inhibiting Histone Deacetylation Prevents Dox-Induced Silencing. A p_{tet}-HPRT bearing cell line was exposed to Dox for 7 days. On day 7, the Dox exposed cells were also exposed to the DNA methylation inhibitor 5-deoxy-azacytidine (AzaC) or the histone deacetylation inhibitor trichostatin A (TSA) and plated in TG 24 hours later. The cells were maintained in TG medium for 12 days and stained. 100,000 cells were plated per dish.

In sum, the results can be interpreted as follows: Reversible gene repression is just that in the vast majority of cells. After the repressing agent (i.e. Dox) is removed from the medium, *HPRT* expression returns in most cells (~ 99.9%), as reflected by the inability of these cells to form clones in the presence of TG. In a subset of cells (~ 0.1%) gene silencing occurs after Dox exposure because these cells can form clones in the presence of TG. Silencing is characterized by promoter region DNA methylation and histone deacetylation, which are common features of tumor suppressor silencing in breast and other cancers. Significantly, our assay allowed us to probe these molecular alterations further and show that histone deacetylation was required for silencing and occurred first and that DNA methylation was not required for silencing and instead occurred secondary to histone deacetylation. These results confirm that silencing is a multi-step process and provide the first system in which silencing can be induced via a well-defined mechanism (i.e., gene repression).

c. *Inducible silencing of the BRCA1 promoter*- Based on the above results, we asked whether the principle of repression-induced silencing could be extended to a genuine tumor suppressor promoter that plays a role in breast cancer. The 218 bp *BRCA1* promoter was chosen because of its obvious importance in breast cancer and its repressibility by hypoxia or exposure to cobalt chloride. *BRCA1* silencing in breast cancer is somewhat atypical as compared with other tumor suppressors because it often occurs in the absence of DNA methylation. The *BRCA1* promoter is also unusual because it is birdirectional and directs expression of an upstream transcript termed *NBR2*.

The *BRCA1* promoter was ligated to the *HPRT* cDNA (Fig. 7) to create p_{BRCA1}-HPRT and mouse cells expressing BRCA1-HPRT were isolated. Two of these cell lines, BSH-2 and BSH-8, were used for the studies described here. The cells were exposed to hypoxic conditions for two weeks and then maintained in hypoxic conditions after TG was added to the medium to select for HPRT deficient clones. Figure 8 shows that TG resistant clones were induced by hypoxic conditions for both cell lines. Once these clones arose, they became hypoxia-independent; in other words hypoxia induced TG resistant clones, but was not required for maintenance of the TG resistant phenotype. We next measured reversion frequencies to determine whether TG resistance was due to silencing as opposed to mutation. Fig. 9 shows that clones tested gave rise to spontaneous revertants and also that TSA exposure increased these reversion frequencies. Both results are consistent with silencing associated with histone deacetylation. Not shown is that 5-aza-dC had no effect on these reversion frequencies and we could find no evidence for *BRCA1* promoter methylation with the bisulfite sequence assay. In total, these results demonstrate that hypoxia induced

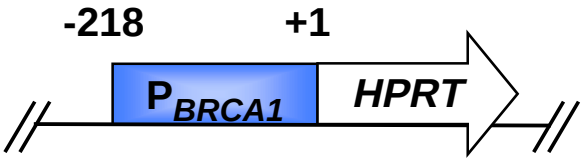


Figure 7. Structure of p_{BRCA1}-HPRT. Construct contains the 218 bp *BRCA1* promoter ligated to HPRT cDNA.

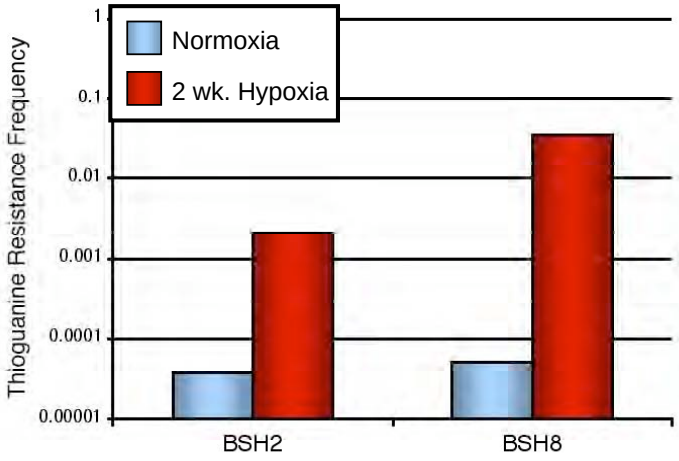


Figure 8. Hypoxia induces loss of expression for *BRCA1* promoter. Two cell lines containing a construct in which the *BRCA1* promoter drives *HPRT* expression were exposed to hypoxia for two weeks and then selected with TG. The TG resistance frequencies were 2-3 orders of magnitude higher under hypoxic conditions. The BRCA1 promoter was obtained from Dr. Peter Glazer (Yale University).

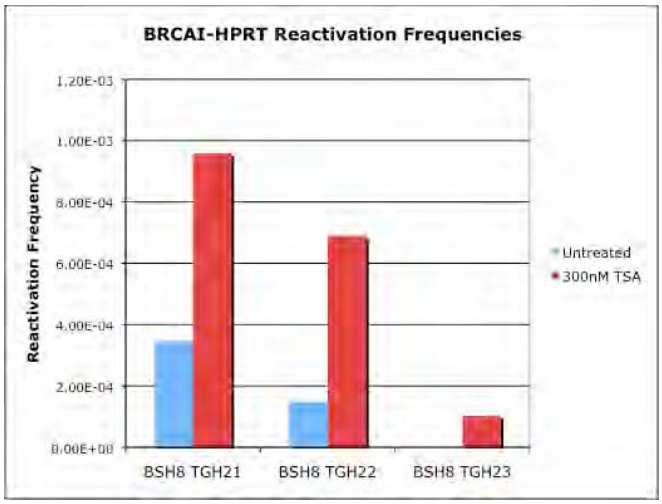


Figure 9. Demonstration that *BRCA1* promoter is silencing in TG resistant clones. Spontaneous (untreated) and TSA-induced reversion frequencies are shown.

silencing of the *BRCA1* promoter and that this silencing was associated with histone modification but not with DNA methylation. We will probe elements of the *BRCA1* promoter, including its capacity for bidirectional transcription, in future work to determine why silencing can occur in the absence of DNA methylation and conversely, how DNA methylation of the silenced *BRCA1* promoter can be initiated.

We also tested cobalt chloride for its ability to induce TG resistant clones and observed a dose response (Fig. 10). TG resistant clones from this experiment are currently being expanded to determine if silencing and/or mutation induced by cobalt chloride are responsible for loss of HPRT expression.

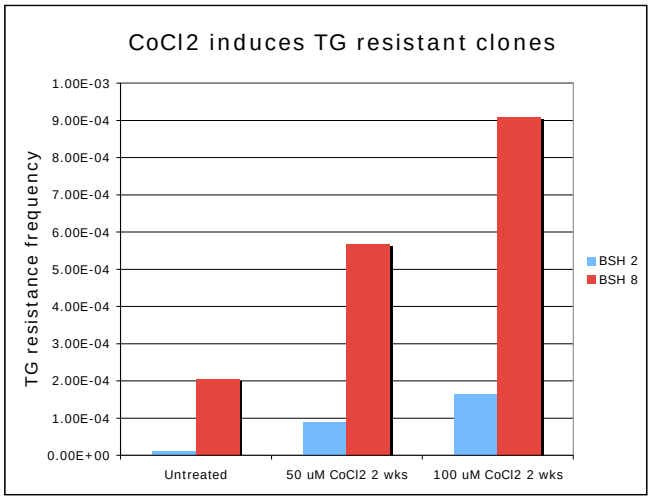


Figure 10. $CoCl_2$ induced TG resistant clones. BSH2 (blue) and BSH8 (red) (see Figure 8) clones containing the p_{BRCA1}-HPRT construct were exposed to 50 or 100 μM $CoCl_2$ for two weeks and then selected in TG for 12 days in the continued presence of $CoCl_2$.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that gene repression can lead to gene silencing in mammalian cells including for the *BRCA1* promoter.
- Demonstrated that silencing is a multi-step process.
- Demonstrated that an early and required step in gene silencing is histone deacetylation.
- Demonstrated that DNA methylation is a secondary step that is not always required for silencing.

REPORTABLE OUTCOMES

- The data obtained from the Concept Award was use to apply for a three-year IDEA Award. The first attempt was unsuccessful, but another attempt will be made in spring 2009.
- We have created cell lines in which gene silencing can be triggered by adding Dox to the medium, and the silencing process can be dissected and studied.
- A manuscript describing the tet-repressible system and silencing is currently in preparation.
- We have created cell lines with a repressible and selectable *BRCA1* promoter.

CONCLUSION- The main significance is that we have demonstrated that gene repression can trigger gene silencing. To the best of my knowledge, these are the first clear demonstrations of a specific trigger for gene silencing. The experimental design is strongly suggestive that environmental repression can induce gene silencing. Thus we have provided the first experimental systems in which gene silencing can be triggered and studied in mammalian cells. An understanding of how silencing is triggered for *BRCA1* and other breast cancer genes can pave the way for preventing this process, which can thereby help to devise therapies to prevent breast cancer.

APPENDICES N/A

SUPPORTING DATA Included above